High-throughput acousto-optic-tunable-filter-based time-resolved fluorescence spectrometer for optical biopsy

Ye Yuan,[1] Ji-Young Hwang,[1] Mowleswaran Krishnamoorthy,[2] Kuntao Ye,[1] Yuan Zhang,[3] Jin Ning,[2] Roy C. Wang,[1] M. Jamal Deen,[3] and Qiyin Fang[1,2,*]

[1]*Department of Engineering Physics, McMaster University, Hamilton, Ontario L854L7, Canada*
[2]*School of Biomedical Engineering, McMaster University, Hamilton, Ontario L854L7, Canada*
[3]*Department of Electrical and Computer Engineering, McMaster University, Hamilton, Ontario L854L7, Canada*
**Corresponding author: qiyin.fang@mcmaster.ca*

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Time-resolved fluorescence spectroscopy has been studied to perform minimally invasive optical biopsy in clinical diagnostics. A critical barrier preventing current time-resolved techniques from clinical studies is their impractically long data acquisition time. We have developed an acousto-optic-tunable-filter (AOTF)-based time-resolved fluorescence spectrometer, which is capable of near real-time data acquisition. Both first-order diffraction beams are collected in this AOTF spectrometer, which results in significantly improved overall throughput. Using standard fluorescence dyes, we have demonstrated that this spectrometer can acquire 200 nm time-resolved spectra within 4 s, while its throughput is comparable to a grating-based spectrometer.

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Endogenous tissue fluorescence spectroscopy has been studied for its potential use as a minimally invasive technique in clinical diagnostics. Steady-state fluorescence techniques are mostly used to measure and analyze the time-integrated fluorescence spectra. For *in vivo* applications, diagnostics signatures based on fluorescence intensity and spectra are often distorted by the presence of multiple fluorophores and chromophores within biological tissue. Compared with steady-state spectrum, fluorescence lifetime is generally independent of artifacts from intensity variation. Providing an additional source of contrast, fluorescence lifetime allows for the differentiation of spectrally overlapped fluorophores with distinct fluorescence decays [1]. Moreover, fluorescence lifetime is sensitive to the microenvironment of fluorophores, enabling the probing of important properties such as pH, binding, and temperature. Recent studies have shown that fluorescence lifetimes and their wavelength dependence may provide important diagnostic information [2,3].

For optical biopsy applications where multiple fluorophores and chromophores are often colocalized, measuring lifetime change as a function of emission wavelength helps to find optimal spectral-temporal signatures for tissue discrimination [4]. Several techniques combining acquisition of fluorescence lifetime and emission spectra have been developed [4–8], but most are too slow for clinical applications. For example, it took a grating-based monochromator 40–60 s to acquire a 200 nm spectrum at 5 nm increment [5] owing to the physical limit of the grating's rotating mechanism; a streak camera coupled to a spectrograph required 15 s for each time-resolved spectrum [4].

We have developed an acousto-optic-tunable-filter (AOTF)-based time-resolved fluorescence spectrometer that is capable of faster data acquisition and has throughput similar to grating-based systems. An AOTF diffracts light at selected wavelength through spatial modulation of the refractive index in a birefringent crystal via acoustic waves induced modulation. There is no moving part in an AOTF, and wavelength switching is achieved by varying the modulation frequency within a few microseconds, which is much faster than grating-based systems (~ms).

In an AOTF, the ordinary and extraordinary polarization components of an incident beam at selected wavelengths are diffracted at opposite sides of the zeroth order (undiffracted) beam. In typical AOTF applications, only one diffracted beam is used, making it inefficient for randomly polarized light. We have designed an AOTF spectrometer that collects both first-order diffracted beams to improve the overall throughput, which is critical for weak, randomly polarized autofluorescence.

Figure 1 shows the schematic of the AOTF-based time-resolved fluorescence spectrometer. A Nd:YAG laser (PNV, Teem Photonics, Meylan, France) provided excitation pulses at 355 nm (300 ps FWHM). A customized bifurcated optical fiber bundle [5] was used to deliver the excitation light to the sample and collect emitted fluorescence, which is collimated using a plano-convex lens before entering into an AOTF device (350–575 nm, TEAF5-0.36-0.52-S, Brimrose, Baltimore, Md.). The maximum rf modulation power of 2 W was used in all experiments reported here. A long-pass filter (LP02-355RU, Semrock, Rochester, N.Y.) was placed at the entrance window of the AOTF to block the backscattered excitation light. Fluorescence emission of narrow wavelength bands was obtained by tuning the AOTF with different modulation frequencies. The zeroth-order beam was blocked by a

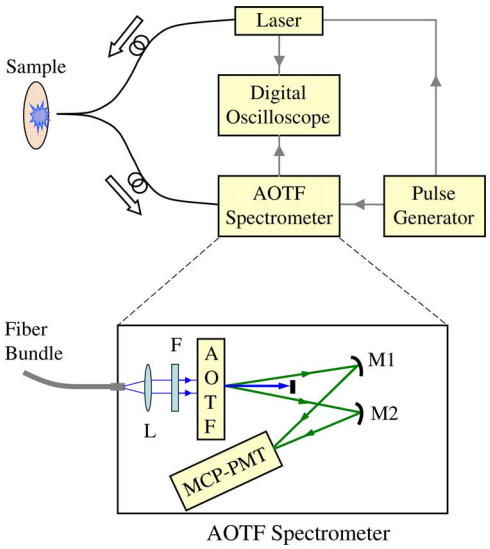

Fig. 1. (Color online) System schematics for the AOTF-based time-resolved fluorescence spectrometer. The inset shows the details of the AOTF setup. L, collimation lens; F, long-pass filter; M1 and M2, concave mirrors.

beam stop, while the two first-order diffracted beams were reflected by two concave mirrors and focused onto a gated microchannel plate photomultiplier tube (MCP-PMT, R5916U-50, Hamamatsu, Iwata-City, Japan). The output signal of the MCP-PMT was amplified using a preamplifier (C5594, Hamamatsu) and digitized by an oscilloscope (2.5 GHz, DPO7254, Tektronix, Beaverton, Oreg.).

Automated data acquisition and synchronization between the light source and the photodetector/oscilloscope were achieved by using a digital pulse generator (9512+, Quantum Composers, Bozeman, Mt.) and a Labview-based program. A photodiode collected scattered excitation light to provide a low jitter trigger to the oscilloscope. Wavelength calibration of the spectrometer was performed by measuring mercury emission lines from a Hg–Ar calibration lamp (HG-1, Ocean Optics, Dunedin, Fla.). The spectral resolution of this spectrometer was measured by scanning the mercury emission lines from the calibration lamp. The spectral bandwidth (FWHM) of the emission lines was measured as 1.6 nm at 365 nm, 3.2 nm at 404.7 nm, 3.3 nm at 435.8 nm, and 4.2 nm at 546.1 nm. Between 360 and 550 nm, the spectral resolution of the spectrometer decreases monotonically from 1.6 nm to 4.2 nm, which is lower than the 0.4–2.2 nm specified by the manufacturer. This decrease in spectral resolution is attributed to the dual-beam configuration of the spectrometer, while the manufacturer's specification was measured using a single beam setup.

To test the performance of the spectrometer, fluorescence emission of 9-cyanoanthracene (Sigma-Aldrich, St. Louis, Mo.) and fluorescein (Sigma-Aldrich) was measured. Both fluorescent dyes were dissolved in ethanol at a concentration of 10^{-4} M. In addition, a mixture solution of 10^{-4} M fluorescein and 10^{-5} M cyanoanthracene was made. Fluorescence decays of the individual and mixed dye solutions were measured from 370 to 570 nm at 5 nm increments within 4 s. Steady-state fluorescence spectra were obtained by integrating fluorescence decay pulses over time, while fluorescence lifetime was calculated for each decay pulse using a multiple exponential deconvolution algorithm [5]. Figure 2 shows the steady-state fluorescence spectra and average lifetimes of the cyanoanthracene, fluorescein, and mixture solutions. In Fig. 2(a), the maximum emissions were at 435 nm for cyanoanthracene and 510 nm for fluorescein. Fluorescence lifetime was found to be monoexponential and remained constant over the emission spectra of both dyes. The lifetimes were 4.09 ± 0.16 ns for fluorescein and 10.8 ± 0.23 ns for cyanoanthracene, which are in good agreement with the literature values [6,9–11]. Owing to the inefficient excitation at 355 nm for the two dyes, relatively high concentration was used and we did not observed significant quenching. The resulting lifetime is in good agreement with literature values. In Fig. 2(b), the steady-state fluorescence spectrum of the mixture solution shows two well-separated emission peaks, which correspond to the maximum emissions of the cyanoanthracene and fluorescein. Average fluorescence lifetimes were also well separated, with longer lifetimes at shorter wavelength representing strong contribution from cyanoanthracene and shorter lifetimes at longer wavelength mainly contributed by fluorescein. These results have demonstrated that this AOTF-based time-resolved fluorescence spectrometer is capable of acquiring both spectral and lifetime information across the entire fluorescence spectra with a short data acquisition time (<4 s). To demonstrate the performance of the spectrometer with biological fluorophores, reduced nicotinamide adenine dinucleotide (NADH, Sigma-Aldrich) and

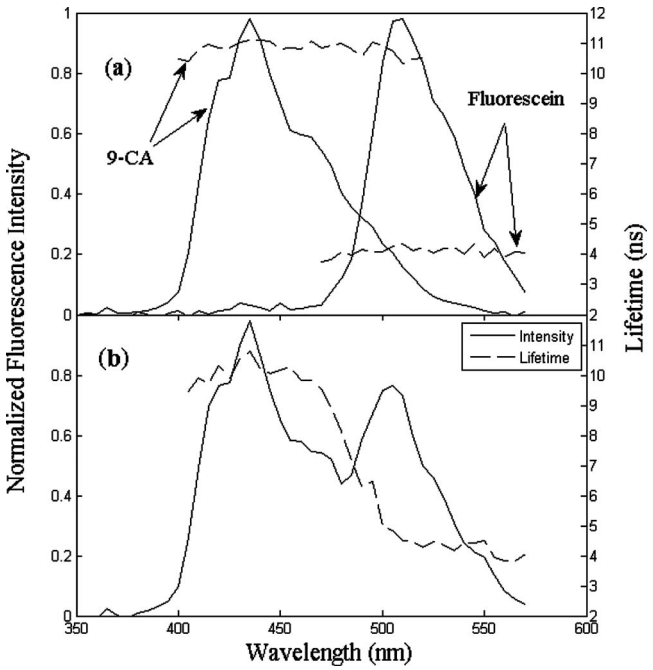

Fig. 2. Time-integrated fluorescence spectra (solid curve) of (a) 10^{-4} M 9-cyanoanthracene (9-CA) and fluorescein solutions and (b) mixture solution of 10^{-4} M fluorescein and 10^{-5} M 9-cyanoanthracene. Average fluorescence lifetimes across the emission spectra are also shown.

flavin adenine dinucleotide (FAD, Sigma-Aldrich) were measured in phosphate buffered saline (pH 7.4) at 10^{-4} M. The fluorescence lifetimes are 0.31 ± 0.02 ns for NADH at 465 nm and 2.47 ± 0.07 ns at 530 nm for FAD, which agree well with previously reported values [5].

Compared with grating-based systems, the light throughput of an AOTF is generally low using the typical single-beam configuration. To improve the overall throughput, we collected both diffracted beams in our AOTF setup. The throughput of the dual-beam AOTF spectrometer was compared with a grating-based monochromator by measuring the collimated laser beam of a pulsed diode laser (LDH-405, PicoQuant, Berlin, Germany) with peak emission at 406 nm. For the grating-based spectrometer, the laser beam was focused onto the entrance slit of the monochromator (M-74100, 1200 l/mm grating blazed at 350 nm, Newport, Stratford, Conn.) via F/#-matched lens. For comparison purpose, the input slit of the monochromator was set at 2.5 mm and the output slit was set at 0.9 mm, which resulted in a spectral bandwidth of 2.8 nm, similar to the AOTF spectrometer. The laser power was measured as 338 μW before entering the spectrometers using an optical power meter (1830-C, Newport), and the output light power was also measured while scanning between 400 and 410 nm at 0.2 nm increments. The results of the throughput tests are shown in Fig. 3, which demonstrated that the dual-beam AOTF spectrometer has comparable throughput to the grating-based spectrometer.

In summary, we have developed an AOTF-based time-resolved fluorescence spectrometer with both fast data acquisition speed and good throughput. The spectrometer is capable of acquiring time-resolved fluorescence across a 200 nm emission spectrum within 4 s. This significantly reduced data acquisition time renders the AOTF spectrometer suitable for near real-time clinical studies. Owing to the exploitation of two diffracted beams, the AOTF spectrometer has nearly doubled the throughput for randomly polarized light, which is comparable with that of grating-based spectrometers. This is a critical improvement for the detection of weak tissue fluorescence emission. Compared with a grating-based system, there are no moving parts in an AOTF spectrometer, which makes it more robust. It should be noted, however, that the spectral resolution of the AOTF spectrometer is low (1–4 nm) and varies with wavelength, which also affect the spectral response of the spectrometer. Spectral intensity calibration is thus required to correct the spectral distortion from the nonuniform spectral resolution. Additionally, the spectral range of the AOTF spectrometer is limited (~200 nm) by the nonlinear crystal compared with the grating-based spectrometer (200–1400 nm). Nevertheless, most tissue endogenous fluorescence is broadband and can be accommodated within this relatively narrow wavelength range. Therefore, the AOTF spectrometer is adequate for optical biopsy applications. Furthermore, this technique may also be applied to fluorescence imaging applications [12] with the use of high sensitivity sensors [13].

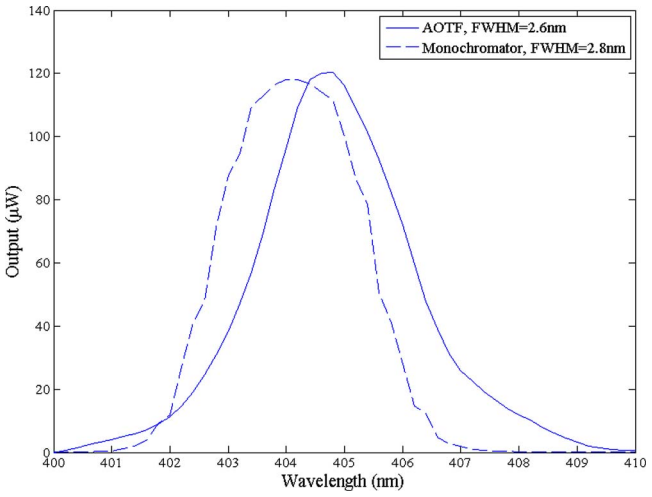

Fig. 3. (Color online) Comparison of the throughputs of the AOTF spectrometer and the grating-based monochromator. The 1 nm difference in their spectral peaks reflects the relatively low spectral resolution of both systems.

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